Spatial Analysis of Almond Leaf Scorch Disease in the San Joaquin Valley of California: Factors Affecting Pathogen Distribution and Spread

R. L. Groves, J. Chen, and **E. L. Civerolo,** USDA-ARS, SJVASC, EIDP, Parlier, CA 93648; **M. W. Freeman,** University of California Cooperative Extension, 1720 S. Maple Ave., Fresno 93702; and **M. A. Viveros,** University of California Cooperative Extension, 1031 S. Mount Vernon Ave., Bakersfield 93307

ABSTRACT

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Almond leaf scorch (ALS) disease has emerged as a serious threat to almond (*Prunus amygdalus*) production areas throughout California's San Joaquin Valley. This disease is caused by the xylem-limited bacterium *Xylella fastidiosa,* and this pathogen is transmitted by xylophagous insects including sharpshooter leafhoppers (Hemiptera: Cicadellidae) and spittlebugs (Hemiptera: Cercopidae). Among four orchards surveyed, enzyme-linked immunosorbent assay (ELISA) and bacterial isolation followed by polymerase chain reaction (PCR) were equally effective in detecting *X. fastidiosa* from ALS-symptomatic trees. Disease incidence varied among almond cultivars in each orchard, with the highest mean incidence and most severe symptoms frequently encountered in 'Sonora'. *X. fastidiosa* isolates consisted of mixtures of grape or "G-genotype" and almond or "A-genotype" strains present in surveyed orchards. The *X. fastidiosa* G-genotypes characterized from each orchard were associated with the most severely affected 'Sonora' trees in three of the four orchards. Both ordinary runs and simple randomization analyses revealed aggregations of ALS in three of the four orchards. Clusters of ALS-affected trees frequently occurred in the outermost orchard rows. Plots of semivariance in ALS incidence over distance varied in shape and magnitude among cultivars. Semivariance increased over distance in 'Sonora' and 'Carmel', indicating spatial dependence or aggregations of incidence best fit by a combination of spherical and linear models. These results document both random and aggregate patterns of ALS spatial distribution in selected orchards and further illustrate how cultivar susceptibility influences the distribution patterns of ALS incidence. Following the recent introduction and establishment of the glassy-winged sharpshooter, *Homalodisca coagulata*, the impact upon the epidemiology and spread of ALS is unknown.

Additional keywords: Pierce's disease

Almond leaf scorch (ALS) disease has emerged recently as a serious threat to almond production areas throughout California's San Joaquin Valley (5,36). This disease is caused by the xylem-limited bacterium *Xylella fastidiosa,* which also causes several plant diseases in California including Pierce's disease (PD) of grape, oleander leaf scorch (OLS), and alfalfa dwarf (AD). *X. fastidiosa* bacterial strains have a diverse host range (5,8,10,30), are genetically diverse, and generally cluster within groups associated with different host species (2,6,7,22,23). Recent studies on the genetic relationships of different *X. fastidiosa* strains support the hypothesis that the bacterial species consists of more than one subspecies or pathovar (5,8,15). Knowledge of the genetic diversity of *X.*

fastidiosa strains associated with ALS in the central San Joaquin Valley of California, especially as it relates to disease epidemiology, is not well understood.

The pathogen is transmitted by xylem-feeding sharpshooters (Cicadellidae) and spittlebugs (Cercopidae) (4,18,28,29,31,33). In California, there are at least 20 species of sharpshooters or spittlebugs capable of transmitting *X. fastidiosa* (31); however, only four species are considered to be epidemiologically important in transmission of *X. fastidiosa* to grapes (18,29). The vector(s) of *X. fastidiosa* associated with ALS, however, has not been well documented. Nevertheless, some leafhopper and spittlebug species have been implicated as possible vectors of *X. fastidiosa*–ALS strains (28,29).

While *X. fastidiosa* has long been present in the San Joaquin Valley of California (24,32), the incidence of ALS appears to have emerged as a significant threat in numerous locations throughout much of the almond-producing region. This increase in ALS is reported to be widely distributed in the affected areas and often appears to be associated with large acreages of adjoining permanent pasture or

irrigated alfalfa forage crops (36). Many vineyards that are recurrently affected by Pierce's disease in this region of the San Joaquin Valley are associated with similar habitats that harbor *X. fastidiosa* vectors (17,29). The spatial pattern of PD incidence in susceptible grape in these areas decreases over distance from inoculum sources and often is associated with the edges of vineyards (16,26,27). Tubajika et al. (35) recently demonstrated that infections of PD were not only aggregated in clusters of diseased vines close to field edges, but were also clustered in disease foci over successive years, suggesting that vine-to-vine, or secondary spread of *X. fastidiosa* by *Homalodisca coagulata* (Say), was an important mechanism for *X. fastidiosa* spread. In coastal California, the blue-green sharpshooter, *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae), is responsible for primary spread of the pathogen from outside inoculum sources in the early spring (14,27).

Limited information is currently available on the distribution and spread of *X. fastidiosa* infections resulting in ALS in the San Joaquin Valley of California. ALS-affected orchards were previously characterized as having few symptomatic trees, which were randomly and widely distributed throughout affected orchards without any association to known vector dispersal habits (28). In recent years, the area-wide incidence of ALS has increased in portions of the southern San Joaquin Valley of California in regions where it has not previously been documented (25,36). Analyses of the spatial patterns of ALS in these newly affected areas will provide new information regarding the relative importance of primary inoculum sources, patterns of *X. fastidiosa* movement into and among susceptible almond cultivars, and the necessity for, or epidemiological importance of, roguing ALS-affected trees.

The objectives of this study were to determine the spatial pattern of ALS incidence in managed almond orchards naturally infected with *X. fastidiosa,* to characterize the patterns of disease spread between ALS genotypes, and to describe the differential patterns of susceptibility to disease among affected almond cultivars.

MATERIALS AND METHODS

Study areas. ALS surveys were conducted at two locations each in Fresno and

Kern counties in the central and southern portions of the San Joaquin Valley of California in 2003. Fresno County orchard 1 was a 24-year-old orchard consisting of almond, *Prunus amygdalus* (Mill.) D.A. Webb (Rosaceae) 'Price', 'Norman', and 'Nonpareil', planted 9.1 m between rows and 7.9 m within rows. The planting pattern consisted of alternating, four-row blocks of cultivars arranged 'Nonpareil', 'Norman', 'Nonpareil', 'Price', respectively, and oriented in an east-west direction. In each four-row block, 'Nonpareil' represented 50% of the trees planted, while 'Norman' (25%) and 'Price' (25%) equally constituted the remainder. Orchard 2 in Fresno County was planted in 1988 and also consisted of alternating, four-row blocks of 'Nonpareil' (50%), 'Sonora' (25%), and 'Carmel' (25%) planted 6.7 m between rows and 7.9 m within rows in an east-west orientation. The two survey orchards in Fresno County were separated by approximately 9.2 km. Kern County orchards 3 and 4 were planted in 1995 and 1996, respectively, and were arranged as alternating, four-row blocks of 'Nonpareil' (50%), 'Sonora' (25%), and 'Fritz' (25%) planted 7.3 m between rows and 6.1 m within rows in a north-south orientation. Survey orchards in Kern County were separated by a distance of approximately 7.7 km.

Disease assessments. Almond leaf scorch disease incidence surveys were conducted 30 October 2003 at the two Fresno County orchards and 7 November 2003 at each of the two Kern County locations. All trees in each orchard were assessed visually for symptoms of ALS, and a symptom severity score was assigned to all trees rated on a scale of 1 to 4: (1 = asymptomatic, healthy trees; 2 = symptoms present on 1 scaffold; 3 = symptoms present on >1 scaffold; and 4 = symptoms on all scaffolds). Trees rated >1 for ALS symptoms were individually marked with colored-flagging for subsequent leaf collections and pathogen detection. Characteristic ALS symptoms often occurred at leaf tips and margins and ranged in appearance from irregular light green or gray-green regions to leaves with tan-colored, marginal scorch that included a characteristic yellow or chlorotic band separating the scorched and green leaf tissues. To confirm the presence of *X. fastidiosa* in ALS-symptomatic trees, approximately five small branch sections (ca. 12.5 cm) with symptomatic leaves were removed from each flagged tree on the date of the survey, placed in labeled plastic bags, and transported in a cooler to the laboratory in Parlier, CA. Leaf samples were stored at 4°C in labeled bags and processed for pathogen isolation and detection within 24 h. Two-dimensional maps of the spatial distribution of *X. fastidiosa*–infected trees were generated for each orchard following diagnostic confirmation.

X. fastidiosa **culture and detection.** Double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) and bacterial isolation attempts followed by polymerase chain reaction (PCR) were used to detect *X. fastidiosa* from trees expressing ALS symptoms. Isolation of *X. fastidiosa* from leaf petioles onto solid media was used to confirm the presence of viable bacteria and for subsequent strain characterization. Leaf petioles were aseptically removed from leaf blades and surface-sterilized in 1% sodium hypochlorite for 2 min followed by three successive rinses in sterile, distilled water. Petioles were then subdivided into three equally spaced regions (each ca. 5 to 7.5 mg), and xylem fluid was expressed aseptically from each petiole section onto the sterile surface of a petri dish using a pair of flame-sterilized, needle-nose pliers. A 25-μl droplet of periwinkle-wilt (PW) broth was added and mixed with the expressed xylem fluid. One 5-μl inoculation loop of the expressed sap mixture was streaked onto periwinkle-wilt solid media modified with Gelrite (PWG) (19) at 28°C, incubated for a period of 10 days, and subsequently held for an additional period not exceeding 40 days. The appearance of opalescent colonies was monitored using a binocular microscope, and candidate isolates were transferred onto new PWG solid media.

Triple-cloned, single colonies were genotyped using primers designed from single nucleotide polymorphisms (SNPs) in the 16S rRNA gene of *X. fastidiosa* using a four-primer PCR format (9). Primer set Temecula 150fc (5′tctaccttatcgtgggggac3′)–Temecula 478rg (3′ccgttaaccaattatggatcaacaa5′) generated a 348-bp DNA amplicon, and primer set Dixon 454fa (5′cctttttgttggggaagaaaa3′)–Dixon 1261rg (3′ctagagcgctcccactcgat5′) produced an 847-bp amplicon. Taken together, the two PCR assays discriminated between both grape and almond genotypes of *X. fastidiosa* by generating an amplicon representative of the *X. fastidiosa* 'Temecula' or an amplicon representative of the *X. fastidiosa* 'Dixon', respectively. Briefly, DNA templates were prepared by suspending a loopful of cell culture transferred from PWG solid media into 100 μl of sterile water. PCR was carried out in a 25-μl reaction volume containing 1 μl of cell suspension (ca. 10 to 25 ng of genomic DNA template) in 1× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$) with the addition of: 0.2 mM of dNTPs, 1 U of *Taq* DNA polymerase (TaKaRa taq, Hot Start Version, Takara Bio Inc., Otsu, Shiga, Japan), and 0.2 μM each of forward and reverse primers. DNA amplification was carried out in an MJ Research Thermocycler (Model PTC-100) programmed with an initial denature at 96°C for 10 min, followed by 30 PCR amplification cycles consisting of denaturing at 96°C for 30 s, annealing at

55°C for 30 s, with a final extension at 72°C for 30 s. A 15-μl volume of each amplification product was separated through a 1.5% agarose horizontal gel electrophoresis at 10 V cm^{-1} for 30 min in 1× TAE buffer (0.1 M Tris-HCl, pH 8.1, 0.2 M glacial acetic acid, and 2 mM EDTA). The gel was stained and visualized with ethidium bromide (0.5 mg ml^{-1}). A 100-bp DNA ladder was used as a size marker, and individual samples were scored as positive for the presence of *X. fastidiosa* if the corresponding DNA band was visualized with UV light.

In addition to bacterial isolation attempts and subsequent PCR, serological assays of petiole tissue samples collected from ALS-symptomatic trees were also conducted using a commercial DAS-ELISA kit (Agdia Inc., Elkhart, IN) according to the instructions provided by the manufacturer. From each symptomatic tree, approximately 50 to 75 mg of leaf petiole tissue was homogenized in 1× phosphate-buffered saline using a Homex 6 tissue extractor (Bioreba Inc., Reinach, Switzerland). One hundred microliters of prepared tissue was collected from each sample and dispensed into replicate wells, and all plates included both positive and negative controls. ELISA results were recorded with a Multiskan MCC/340 microplate reader (ThermoLabsystems Corp., Vantaa, Finland) at a wavelength of 490 nm. Plants were scored positive for *X. fastidiosa* if the optical density (OD) of test wells was greater than that of the mean OD of noninoculated, healthy control plants of the same plant cultivar plus 3 standard deviations.

Data analyses. Almond leaf scorch disease incidence analyses were completed using SAS, version 7 (SAS Institute, Cary, NC). Methods of *X. fastidiosa* detection including DAS-ELISA and bacterial isolation were compared within and among almond cultivars at each surveyed orchard location using a χ^2 analysis of positive ratios for ALS symptomatic tissues. Mean ALS disease incidence, ALS disease severity rating, and mean proportions of *X. fastidiosa* genotypes were compared among almond cultivars at each experimental orchard using a randomized, stripped plot analysis of variance, general linear models procedure (PROC GLM; LSMEANS) using the repeating four-row planting pattern as experimental replicates. In Fresno County, orchards 1 and 2 contained 32 and 16 rows, respectively, resulting in 8 and 4 experimental replicates; whereas orchards 3 and 4 in Kern County contained 64 and 56 rows, resulting in 16 and 14 experimental replicates. Comparisons of mean ALS disease incidence and disease severity rating were conducted after an initial log$_{10}$ data transformation for normalization of variance and means separated using PROC GLM (LSMEANS). Comparisons of the mean proportion of *X. fastidiosa* genotypes

among almond cultivars were initially arcsine square transformed and means separated by F tests (α = 0.05). All means presented in tables and figures were back-transformed.

For each survey location, ALS distribution was evaluated within and across cultivars using a distribution free test, or ordinary "runs" analysis, to test for clustering or a random distribution of diseased trees (21). The analyses were conducted by cultivar (within rows) and across cultivars (across rows) for each row or column on the respective disease assessment dates. The percentage of rows with clustered patterns of disease was calculated, and the location of rows or columns with clustered patterns of ALS was noted. Only ALS-symptomatic trees testing positive for the presence of *X. fastidiosa* by either DAS-ELISA or bacterial isolation on solid media were used in this and the following distribution analyses.

To test for "edge effects" in the distribution of ALS-affected trees, a simple permutation procedure was constructed (34). Each orchard was first subdivided into equal-sized quadrants consisting of 4 adjacent rows (all cultivars represented) of 4 trees per row. A new variable (I_d) was then generated for each quadrant as the sum of the observed disease incidence plus one (I_n + 1) multiplied by the distance (d_m) in meters from a particular field edge. Next, disease incidence data were randomly rearranged and quadrant products summed for 500 separate iterations relative to each of the four field edges in each orchard. The null hypothesis evaluated in this test, that ALS incidence is randomly distributed within an affected orchard and not associated with a particular field border, can then be rejected if the true test statistic (ΣI_d) is less than 95% of the random values generated for each respective field border. Tests were constructed and analyzed by cultivar individually and across cultivars simultaneously relative to field borders.

Geostatistical analysis of ALS within fields. Latitude and longitude coordinates of the four experimental orchards initially were transformed into plane coordinates using a Universal Transverse Mercator (UTM) projection in ArcView, Geographic Information Systems (GIS), Version 3.3 (Environmental Systems Research Institute, Redlands, CA). The UTM coordinates were used to conduct empirical semivariogram analyses on the spatial patterns of ALS incidence, disease severity, and *X. fastidiosa* genotype in each orchard and among cultivar within orchards. Plots of modeled covariance lines were calculated as:

$$\gamma(h) = \left[1\big(2\big|n(h)\big|\big)^{-1}\right] \sum_{i=1}^{n(h)} (z_i - z_{i+h})^2$$

and represent the average of squared differences in values z between grid cells i and j separated by a lag distance h. The plot of $\gamma(h(j))$ against distance h over all directions results in an omnidirectional semivariogram that typically increases in value with increasing distance (11,37). Parameters used to characterize the semivariogram plot include the nugget, which represents the value of $\gamma(h)$ at $h = 0$ or measurement error; the range, or sample distance between points beyond which little or no autocorrelation among variables occurs; and the sill, which corresponds to the overall variance for data greater than the range. Semivariogram shapes were fit to circular, exponential, linear, and spherical models using a nonlinear, least squares optimization weighted by the number of distance pairs. The best model fit was selected based upon the lowest error mean square value. The degree of anisotrophy, or directional spatial dependence, was examined for ALS incidence, disease severity rating, and genotype within rows (zero degrees azimuth) and across rows at directions of 22.5, 45, 67.5, 90, 112.5, 135, and 157.5° azimuth.

RESULTS

***X. fastidiosa* detection.** *X. fastidiosa* was diagnosed in each of the four orchards surveyed in Fresno and Kern counties where ALS symptoms were observed. Averaging over the four surveyed orchards, DAS-ELISA and bacterial isolation on solid media were equally effective (χ^2 = 0.47, df = 1, P = 0.7339) in detecting *X. fastidiosa* from ALS-symptomatic trees, averaging 67.3% (134/199 attempts) and 56.7% (94/166 attempts) positive detection, respectively. In Fresno County orchard 1, *X. fastidiosa* was detected more frequently by DAS-ELISA than by bacterial isolation in almond cultivars 'Price'

and 'Norman' (χ^2 = 8.904, df = 1, P = 0.0094; χ^2 = 6.77, df = 1, P = 0.0188, respectively), whereas no differences in detection frequencies were observed in 'Nonpareil' (χ^2 = 2.66, df = 1, P = 0.2013) (Table 1). In Fresno County orchard 2, both methods were equally efficient in detecting *X. fastidiosa* within 'Sonora' (χ^2 = 2.14, df = 1, P = 0.2891) and 'Nonpareil' (χ^2 = 2.37, df = 1, P = 0.2331), and again in Kern County orchard 3, detection frequencies were similar among the three cultivars, 'Sonora', 'Fritz', and 'Nonpareil' (χ^2 = 1.75, df = 1, P = 0.3818; χ^2 = 1.86, df = 1, P = 0.3218; χ^2 = 1.19, df = 1, P = 0.4539, respectively) (Table 1). In Kern County orchard 4, both methods were comparable for 'Sonora' (χ^2 = 1.24, df = 1, P = 0.4011) and 'Fritz' (χ^2 = 5.18, df = 1, P = 0.1318) (Table 1), and no 'Nonpareil' trees were observed with ALS symptoms. Three asymptomatic trees of each almond cultivar were selected randomly from each orchard for serological analyses and isolation on solid media. Neither detection method yielded a positive assay for the presence of *X. fastidiosa* among 36 samples collected from asymptomatic almond cultivars over all survey locations (χ^2 = 0.0, df = 1, P = 1).

Disease assessments. Incidence of ALS disease varied among cultivars in each of four surveyed orchards in Fresno and Kern counties in California. Among 709 trees in Fresno County orchard 1, 32 trees had characteristic ALS symptoms. Combining the results of both ELISA and bacterial isolation, *X. fastidiosa* was confirmed in 19 (1.6%) symptomatic trees. Incidence varied more (F = 8.77, df = 2, 14, P = 0.0110) among cultivars with higher disease incidence ('Norman' [2.2%] and

Table 1. Comparative frequencies of *Xylella fastidiosa* detection in almond leaf scorch–symptomatic cultivars using double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) and isolation on solid periwinkle wilt (PW) media

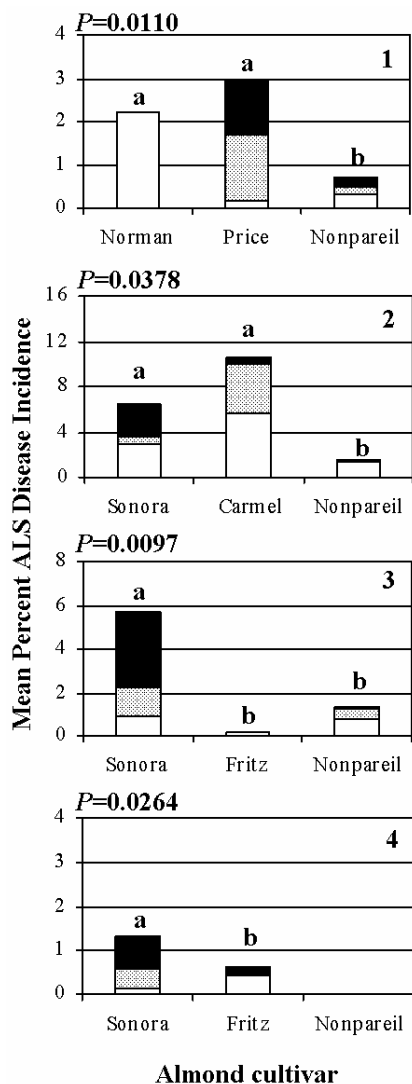| County	Location	Cultivar	DAS-ELISA[w]	*X. fastidiosa* isolation[x] (no.+/total)
Fresno	Orchard 1	'Price'	8/14 (57.1%) a[y]	4/14 (28.6%) b
		'Norman'	6/14 (42.9%) a	3/14 (21.4%) b
		'Nonpareil'	3/4 (75.0%) a	2/4 (50.0%) a
	Orchard 2	'Sonora'	11/16 (68.8%) a	9/16 (56.3%) a
		'Carmel'	21/26 (80.7%)	N/A[z]
		'Nonpareil'	6/11 (54.6%) a	4/11 (36.4%) a
Kern	Orchard 3	'Sonora'	45/62 (72.6%) a	49/62 (79.0%) a
		'Fritz'	2/5 (40.0%) a	1/5 (20.0%) a
		'Nonpareil'	20/30 (66.7%) a	17/30 (56.7%) a
	Orchard 4	'Sonora'	8/11 (72.7%) a	7/11 (63.6%) a
		'Fritz'	4/6 (66.7%) a	2/6 (33.3%) a
		'Nonpareil'	0	0

[w] Optical densities at 492 nm ranged between 0.108 and 0.577 in *X. fastidiosa*–infected tissue and between 0.059 to 0.088 in asymptomatic, healthy tissue. Plant tissue was considered *X. fastidiosa*–infected if the optical density of test wells exceeded the mean optical density of asymptomatic controls of the same plant variety plus 3 standard deviations.

[x] Early passage, single colonies isolated on PW solid media were confirmed as *X. fastidiosa* using primers designed from single nucleotide polymorphisms in the 16S rRNA gene.

[y] Detection frequencies not followed by the same lowercase letter within rows are significantly different, while frequencies not followed by the same uppercase letter in columns by orchard are significantly different by χ^2 analyses (α = 0.05).
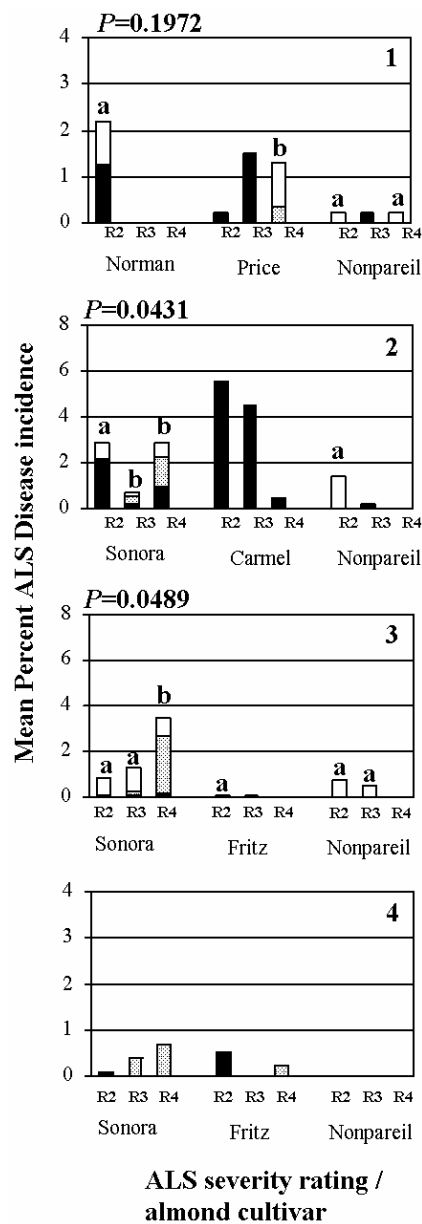
[z] N/A: not available; petiole samples were not collected from 'Carmel' for bacterial isolation assays and are not reported.

'Price' [2.9%)) than in 'Nonpareil' (0.5%) (Fig. 1A). Similarly, the proportion of *X. fastidiosa*–infected trees classified in three disease severity categories differed among cultivars ($F = 8.03$, df = 4, 23, $P = 0.0099$). Affected 'Norman' trees were the least severely affected (rating = 2), while nearly all 'Price' trees had ALS severity ratings 3 (50%) and 4 (43%). The few 'Nonpareil' trees were distributed nearly equally among the three disease rating categories. In Fresno County orchard 2, 53 trees exhibited ALS symptoms. *X. fastidiosa* was confirmed in 41 (5.8%) of the symptomatic trees, and disease incidence differed

($F = 7.81$, df = 2, 8, $P = 0.0378$) among cultivars, with the highest incidence in 'Carmel' followed by 'Sonora' and 'Nonpareil', averaging 10.5, 6.5, and 1.6%, respectively (Fig. 1B). The proportion of *X. fastidiosa*–infected trees in each severity category did not differ ($F = 3.82$, df = 4, 13, $P = 0.0773$) among almond cultivars in orchard 2. Nearly all 'Nonpareil' trees

(>90.0%) were rated in the least severe category (disease rating = 2).

In Kern County orchard 3, 97 trees possessed characteristic ALS symptoms, from which *X. fastidiosa* was confirmed in 75 (2.1%) trees. Incidence among cultivars differed ($F = 10.09$, df = 2, 28, $P = 0.0097$), with the highest incidence recorded in 'Sonora' (5.7%) followed by 'Nonpareil' (1.3%) and 'Fritz' (0.2%) (Fig. 1C). The proportion of trees classified in each severity category varied among cultivars ($F = 13.66$, df = 4, 26, $P = 0.0103$), with 62% of all 'Sonora' classified in the highest disease rating category and the remaining trees rated in categories 2 (15%) and 3 (23%). All *X. fastidiosa*–infected 'Fritz' trees surveyed were rated in the least severe category, whereas the majority (>90%) of 'Nonpareil' rated in severity categories 2 (61%) and 3 (36%). In Kern County orchard 4, 17 trees exhibited characteristic ALS symptoms from which *X. fastidiosa* was confirmed in 13 trees (0.5%). Incidence of *X. fastidiosa* differed ($F = 5.09$, df = 2, 26, $P = 0.0264$) among cultivars (Fig. 1D), with the highest incidence observed in cultivar 'Sonora' (1.3%) followed by 'Fritz' (0.6%). No ALS-symptomatic trees were observed in 'Nonpareil' at orchard 4. The proportion of *X. fastidiosa*–infected trees in each disease severity category did not differ between cultivars ($F = 3.61$, df = 1, 26, $P < 0.1954$) 'Sonora' and 'Fritz'.

***X. fastidiosa* genotype relationship to ALS incidence.** Mixtures of *X. fastidiosa* genotypes were observed in three of the four surveyed orchards. Almond genotypes (A-genotype) were the most prevalent (70%) among positive isolations ($N = 55$), whereas only 30% ($N = 23$) were documented as grape-genotypes (G-genotype). In Fresno County orchard 1, all positive isolations obtained from cultivars 'Norman' ($N = 3$) and 'Nonpareil' ($N = 2$) were A-genotypes, whereas one of four 'Price' isolates was G-genotype (Fig. 2A). No differences ($F = 4.76$, df = 1, 2, $P = 0.1972$) in genotype proportions were observed among almond cultivars at this location. In orchard 2, the proportion of *X. fastidiosa* genotypes varied ($F = 322.68$, df = 1, 2, $P = 0.0327$) among almond cultivars (Fig. 2B). Only A-genotypes were associated with 'Nonpareil', whereas 'Sonora' contained a mixture of both A- ($N = 5$) and G-genotypes ($N = 4$). Within 'Sonora' alone, genotype proportions varied ($F = 9.84$, df = 1, 4, $P = 0.0231$) among severity ratings where all symptomatic trees rated 2 were characterized as A-genotype and 50 and 67% of trees rated 3 and 4 were classified as G-genotypes, respectively. In Kern County, a mixture of G- ($N = 18$) and A-genotypes ($N = 49$) occurred in orchard 3, and the relative proportion of both genotypes differed ($F = 167.83$, df = 1, 2, $P = 0.0489$) among cultivars (Fig. 2C). All 18 G-genotypes (27%)

Fig. 1. Mean incidence of almond leaf scorch (ALS) among almond cultivars in orchards in Fresno (1 and 2) and Kern (3 and 4) counties in California illustrating the proportion of ALS-affected trees in each disease category: (open bar = R2, symptoms present on 1 scaffold; gray bar = R3, symptoms present on >1 scaffold; and black bar = R4, symptoms on all scaffolds). Probabilities of a difference in mean ALS incidence among almond cultivars at each orchard are provided ($P = 0.05$) (PROC GLM). Column means by variety with dissimilar letters are significantly different by PROC GLM, LSMEANS ($\alpha = 0.05$).

Fig. 2. Mean incidence of almond leaf scorch (ALS) within almond cultivars in orchards in Fresno (1 and 2) and Kern (3 and 4) counties in California illustrating the proportion of A-genotypes and G-genotypes of *Xylella fastidiosa* (open bar = A-type; gray bar = G-type; and black bar = unclassified) in each disease category (R2 = symptoms present on 1 scaffold; R3 = symptoms present on >1 scaffold; and R4 = symptoms on all scaffolds). Probabilities of a difference in the proportion of *X. fastidiosa* genotypes among cultivars are provided ($P = 0.05$) (PROC GLM). Column means by orchard with dissimilar letters contain significantly different *X. fastidiosa* genotype proportions by PROC GLM, LSMEANS ($\alpha = 0.05$).

occurred in 'Sonora', whereas only A-genotypes were isolated from both 'Fritz' (N = 1) and 'Nonpareil' (N = 17). In 'Sonora', the proportion of genotypes again differed (F = 8.13, df = 1, 12, P = 0.0109) among severity ratings. In orchard 4, all positive *X. fastidiosa* isolations were characterized as G-genotypes in 'Sonora' (N = 9) and 'Fritz' (N = 2) (Fig. 2D).

Spatial patterns of ALS. Ordinary runs analyses revealed aggregations of *X. fastidiosa*–infected trees in three of the four almond orchards surveyed in 2003. Among the four orchards surveyed, the proportion of disease aggregates within rows of specific cultivars was determined to be greater than the proportion of aggregates across rows. In survey orchard 1, the proportion of rows with significant (α = 0.05) aggregations ranged between 0.12 and 0.06 with clusters detected in 'Price' and 'Norman', respectively (Table 2). The clustering of *X. fastidiosa*–affected trees in 'Price' was present in two orchard rows (rows = 25, 29) and in 'Norman' in only a single row (row = 27). All aggregates were located within five of the outermost rows of the surveyed orchard along the orchard's southern border (Fig. 3A). No significant aggregations of diseased trees were detected within rows of 'Nonpareil', which had the lowest overall incidence, nor were any disease clusters noted across rows. In orchard 2, the frequency of occurrence of *X. fastidiosa* clusters within rows ranged between 0.20 and 0.40 for 'Sonora' and 'Carmel', respectively (Table 2). Clusters of affected 'Sonora' trees were detected in only a single row (row = 3), which corresponded to the outermost row oriented parallel to the northern field boundary (Fig. 3B). Disease aggregates within rows of 'Carmel' were also marginally distributed and associated with outer orchard rows parallel to both northern (row = 14) and southern (row = 2) boundaries (Fig. 3B). Significant aggregations of *X. fastidiosa*–infected trees across rows were observed in the two outermost rows (rows = 1, 2) along the orchard's western boundary (Table 2).

Clusters of *X. fastidiosa*–infected trees were detected along field boundaries in Kern County orchard 3. In 'Nonpareil' and 'Sonora', the frequency of rows containing disease clusters ranged between 0.04 and 0.24, respectively (Table 2). Disease aggregates within rows included the first four 'Sonora' rows parallel to the western field boundary (rows = 1, 5, 9, 13) and only a single 'Nonpareil' row (row = 20) located closer to the field interior. No significant aggregations of diseased trees were observed within rows of 'Fritz'. Across row aggregations of *X. fastidiosa*–infected trees were detected among four of the six southernmost border rows (rows = 59, 60, 61, 62) in Kern County orchard 3 (Fig. 3C). Orchard 4 in Kern County had the lowest overall ALS incidence among each of the

four selected orchard locations, and no within or across row clusters of diseased trees were detected.

The spatial patterns of *X. fastidiosa*–infected trees relative to specific orchard boundaries were investigated further using a simple permutation, or randomization procedure. Similar to the results obtained from the ordinary runs analysis, a significant edge effect was observed in 'Price' in 22 out of 500 (P = 0.044) iterations of the approximate randomization procedure associated with the southern orchard boundary in Fresno orchard 1 adjoining currently nonirrigated, fallow land (Fig. 3A). In Fresno orchard 2, the true ALS test statistic for 'Sonora' was significantly different (P = 0.03) among 13 of 500 randomized estimates relative to the eastern orchard boundary, which adjoined approximately 2.8 ha of irrigated, permanent pasture (Fig. 3B). At the same orchard, 27 of 500 approximate randomizations resulted in a significant (P = 0.05) western edge association observed in 'Carmel', which adjoined a larger (ca. 22.8 ha) set of subdivided, irrigated permanent pasture blocks (Fig. 3B). Diseased trees were marginally distributed in Kern County orchard 3, where 25 of 500 iterations of the randomization procedure resulted in a significant (P = 0.05) edge effect in 'Nonpareil' associated with the southern border of the orchard adjacent to irrigated alfalfa (Fig. 3C). No significant edge effects were observed in orchard 4 of Kern County using the approximate randomization procedure. Including all almond cultivars simultaneously in the randomization, no significant ALS edge effects were observed relative to orchard boundaries at any of the four survey orchards.

Geostatistical analysis of ALS distribution. Plots of semivariance in ALS incidence over distance varied in shape and magnitude among cultivars individually and across cultivars simultaneously at each of the four survey locations (Fig. 4). In Fresno County orchard 2, where ALS incidence was highest (6.5%), semivariance increased over distance in 'Sonora' and 'Carmel', indicating spatial aggregations of ALS incidence (Fig. 4B). The shape of the 'Sonora' semivariogram was best fit by a spherical model with an estimated sill and effective range value of 7.42 and 39.17 m, respectively (Table 3). A linear model best fit the 'Carmel' semivariance plot with a lower estimated sill value of 4.91 and an increasing, nonzero slope (P = 0.04). Averaging over all cultivars simultaneously, semivariance increased over distance and was best fit to a spherical model with an estimated sill of 2.04 and an effective range of 27.57 m (Table 3). No detectable spatial dependence was observed in 'Nonpareil' in orchard 2. Spatial aggregations of ALS incidence were observed in the cultivar 'Sonora' and across all cultivars simultaneously in Kern County orchard 3 (Fig. 4C). In both instances, spherical models best approximated the semivariance plots over distance with sill estimates of 3.07 and 2.09, respectively, and corresponding range values of 31.88 and 19.65 m (Table 3). No spatial dependence in ALS incidence was observed at either of the remaining orchards (1 and 4) in Fresno and Kern counties where the lowest mean disease incidences were recorded (1.9 and 0.6%, respectively) (Table 3). Because no directional variograms differed from omnidirectional semivariance plots with respect

Table 2. Ordinary runs analysis of almond leaf scorch (ALS) disease aggregations in selected almond orchards of Fresno and Kern counties, CA

County	Orchard	Cultivar	ALS incidence	Proportion of tests with significant Z value (aggregate rows of ALS-infected trees)	
				Within rows[y]	Across rows[z]
Fresno	Orchard 1	'Price'	0.029	0.12 (25, 29)	
		'Norman'	0.022	0.06 (27)	0.00
		'Nonpareil'	0.005	0.00	
	Orchard 2	'Sonora'	0.065	0.20 (4)	
		'Carmel'	0.105	0.40 (2, 14)	0.04 (1, 2)
		'Nonpareil'	0.016	0.00	
Kern	Orchard 3	'Sonora'	0.057	0.24 (1, 5, 9, 13)	
		'Fritz'	0.002	0.00	0.06 (59, 60, 61, 62)
		'Nonpareil'	0.013	0.04 (20)	
	Orchard 4	'Sonora'	0.013	0.00	
		'Fritz'	0.006	0.00	0.00
		'Nonpareil'	0.000	…	

[y] Reported values are the proportion of tests with significant aggregations of ALS-affected trees by almond cultivar within rows (α = 0.05). Parenthetical values indicate the within-row position(s) where significant ALS aggregations were observed. Almond cultivars in Fresno County orchards (1 and 2) were planted in a (west-east) orientation with ascending row numbers (north–south) and ascending across-row columns (west-east). Kern County orchards (3 and 4) were planted in a (north-south) orientation with corresponding ascending row numbers (west-east) and ascending across-row columns (north-south).

[z] Reported values are the proportion of tests with significant aggregations averaging among cultivars across rows (α = 0.05). Parenthetical values indicate across-row position(s) of significant ALS aggregations.

to ALS incidence, only omnidirectional plots are illustrated.

Few spatial aggregates of ALS severity rating and *X. fastidiosa* genotype were detected among the four orchards surveyed. In orchards 2 and 3 of Fresno and Kern counties, where ALS incidence was greatest, spatial dependence of *X. fastidiosa*–infected 'Sonora' trees rated in the highest disease severity category (rating = 4) was observed. Semivariance plots of these 'Sonora' trees over distance were best fit to spherical and linear models at orchards 2 and 3, respectively (Table 3). Semivariance gradually increased at or-

chard 2 to an estimated sill value of 0.49 with a corresponding range estimate of 24.77 m. In orchard 3, a linear model best fit the steadily increasing semivariance over distance ($P = 0.0239$) and possessed a higher estimated sill value of 1.43 (Fig. 5). Spatial dependence among *X. fastidiosa* G-genotypes was observed at orchard 3 in Kern County, whereas no corresponding A-genotype aggregations were detected (Fig. 6). Specifically, semivariance plots of the less frequently occurring G-genotype of *X. fastidiosa* were best fit to an exponential model where semivariance rose sharply to an estimated sill value of 0.98

with an estimated range not exceeding 18.33 m (Table 3). Directional plots of semivariance did not differ from omnidirectional plots and are not illustrated for either ALS symptom severity or *X. fastidiosa* genotype.

DISCUSSION

X. fastidiosa was isolated regularly from leaf petioles of trees exhibiting ALS symptoms in each of the surveyed orchards. Detection frequencies were similar using ELISA and bacterial isolation techniques with the exception of only a single survey location where methods differed somewhat between two of the cultivars surveyed. Such differences may result more from *X. fastidiosa* distribution patterns within affected almond trees than from the relative sensitivity or efficiency of either assay. Although little is known about the colonization patterns of *X. fastidiosa* in almond, recent work by Almeida and Purcell (4) demonstrated that bacterial populations in experimental and naturally infected almond were 10- to 100-fold lower than populations in susceptible grape. They proposed that xylem vessel structure may, in part, limit the uniform distribution of the pathogen within the trees, and prolonged infections may be required for widely disseminated infections to result. Similarly, Mircetich et al. (24) observed that the colonization rates (10 to 15%) of xylem vessels in affected almond were considerably less in comparison to infected grape, where nearly 20% of vessels were occluded.

Differences in ALS susceptibility among almond cultivars have been observed previously (25,32). Historically, incidence was most severe in cultivars 'Long IXL', 'IXL', and 'Jordanolo' with characteristic foliar leaf scorching symptoms, stem pitting on many secondary branches, and a large proportion of dead spurs followed by dieback of terminal growth. More recently, additional almond cultivars have been added to a growing list of ALS-susceptible cultivars and include 'Sonora', 'Peerless', and an increasing number of affected 'Nonpareil' (36). The incidence of ALS in other cultivars, including 'Butte', 'Mission', 'Aldrich', and Padre', has been reported to be rare or very infrequent (25,32). In the present study, differences in ALS incidence and severity were observed among the cultivars examined. Hopkins (20) demonstrated that virulence in Pierce's disease strains of *X. fastidiosa* was associated with increased bacterial populations. More recently, it has been determined that for grape strains of *X. fastidiosa* that were pathogenic to both grapes and almonds, the number and distribution of living bacterial cells within plants can be influenced greatly by host plant species (3,12). In a recent study, Almeida and Purcell (4) observed variable populations of *X. fastidiosa* in ALS-affected trees among
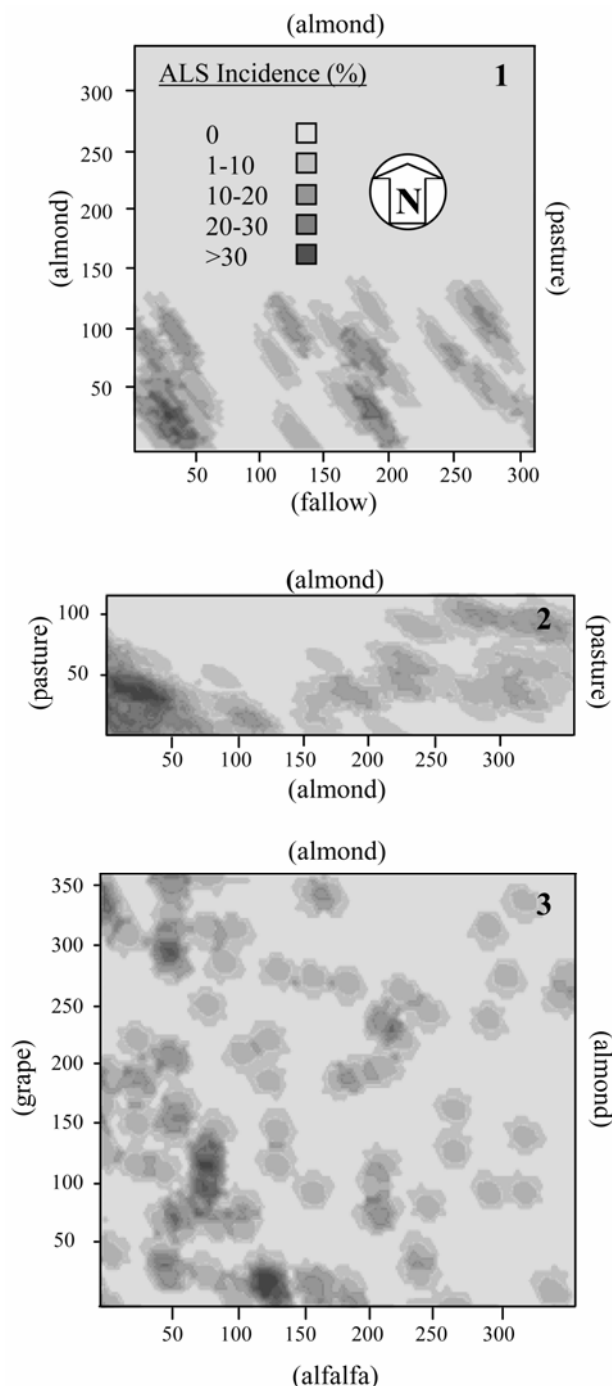
Fig. 3. Distribution of almond leaf scorch (ALS) incidence using universal kriging interpolations illustrating adjoining land uses at survey orchards in Fresno (1 and 2) and Kern (3) counties in California.

five field locations sampled in 2002 ranging between 1.3×10^5 and 9.5×10^7, although no information was provided about the particular cultivars evaluated. In our study, variations in ALS incidence and disease severity among cultivars may be influenced by differences in bacterial populations present in each almond cultivar.

Observed differences in ALS incidence among cultivars may be linked partially to infection by specific *X. fastidiosa* genotypes. In our study, the largest proportion of *X. fastidiosa*–infected trees was observed in 'Sonora' ($N = 73$). Among these infected trees, over 41% ($N = 30$) were infected by the G-genotype of *X. fastidiosa*. Earlier surveys of ALS throughout central and northern portions of California's Central Valley reported the disease to be only sporadic and widely distributed (28), and the emergence of the disease into southern portions of the Central Valley has been considered quite recent (5,36). Moreover, the occurrence of grape genotypes isolated from susceptible almond in this region of the San Joaquin Valley was originally reported to be only a rare occurrence due to accidental infections by infective vectors (28). Our field survey results illustrate that G-genotypes of *X. fastidiosa* were encountered regularly in orchards within the region and may help to explain the observed increase in disease incidence among susceptible cultivars.

The distribution and abundance of ALS in affected almond orchards of California has not been well documented and has previously been characterized as often low in overall incidence with few symptomatic trees scattered randomly or in patches in a manner inconsistent with the dispersal habits of known insect vectors (4). Further, disease progress within affected orchards was characterized as slow, impacting the productivity of individual orchards in 10 to 15 years (24,32). The spatial distribution patterns and movement of *X. fastidiosa* resulting in Pierce's disease of susceptible grapes has been well documented in California (18,27,35) and the southeastern United States. (1). Patterns of PD distribution in the north coast vineyards of California result from immigrating populations of the blue-green sharpshooter, *G. atropunctata* (27). In this important wine grape region of California, Pierce's disease incidence has been described as marginally distributed along field borders or riparian buffers resulting primarily from the spread of *X. fastidiosa* into vineyards from outside inoculum sources with only very limited secondary spread of the pathogen. In
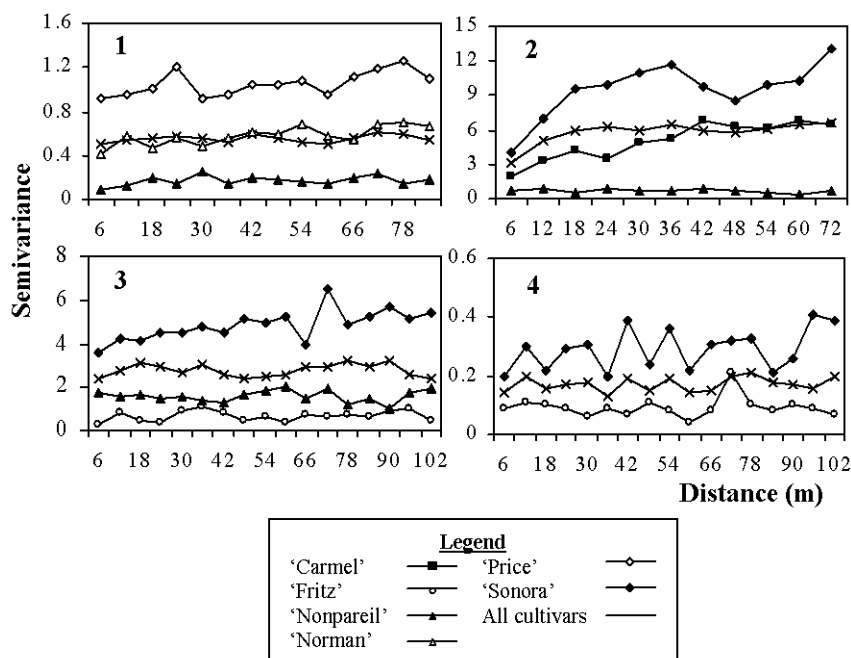
Fig. 4. Semivariogram plots of almond leaf scorch incidence in Fresno (1 and 2) and Kern (3 and 4) county survey orchards depicting spatial dependence among all almond cultivars and each cultivar independently.

Table 3. Semivariogram attributes and selected model parameters of almond leaf scorch (ALS) disease incidence, severity rating, and *Xylella fastidiosa* genotype in surveyed almond orchards of Fresno and Kern counties in California, 2003

Variable	County	Orchard	Almond cultivar	Model[w]	Semivariogram parameters[v]			R^2	P^x
					C_0	C_1	a (m)		
Incidence	Fresno	1	'Price'	Linear	0.97	1.08	…	0.79	0.1338
			'Norman'	Linear	0.44	0.52	…	0.88	0.1901
			'Nonpareil'	Linear	0.19	0.23	…	0.93	0.5387
			All	Linear	0.52	0.58	…	0.90	0.6991
		2	'Sonora'	Spherical	2.91	7.42	39.17	0.85	…
			'Carmel'	Linear	1.83	4.91	…	0.84	0.0421
			'Nonpareil'	Linear	0.54	0.69	…	0.91	0.6188
			All	Spherical	2.04	5.52	27.57	0.77	…
	Kern	3	'Sonora'	Spherical	3.07	4.27	31.88	0.69	…
			'Fritz'	Linear	0.61	0.67	…	0.86	0.0976
			'Nonpareil'	Linear	1.63	1.55	…	0.81	0.4713
			All	Spherical	2.09	2.41	19.65	0.64	…
		4	'Sonora'	Linear	0.26	0.28	…	0.79	0.2279
			'Fritz'	Linear	0.1	0.11	…	0.83	0.7345
			All	Linear	0.15	0.16	…	0.90	0.5557
Rating[y]	Fresno	2	'Sonora'	Spherical	0.11	0.48	30.72	0.89	…
	Kern	3	'Sonora'	Linear	0.28	1.47	…	0.85	0.0239
Genotype[z]	Kern	3	'Sonora'	Exponential	0.09	0.98	18.33	0.81	…

[v] C_0 = experimental error or the semivariance at the $\gamma(h)$ intercept (lag distance = 0); C_1 = estimated sill value or covariance estimate which remains unchanged with increasing distance (h); a = range value or distance (h) beyond which covariance remains unchanged.

[w] Omnidirectional, semivariance plots fit to the best linear or nonlinear model using a weighted, least-squared analysis and model selection based on lowest error mean square.

[x] Overall F test probability values ($\alpha = 0.05$) for H_o: slope = 0.

[y] Omnidirectional semivariance plots for ALS severity rated trees classified as rating = 4.

[z] Omnidirectional semivariance plots for ALS G-genotypes.

Florida, where the highly mobile and polyphagous glassy-winged (*H. coagulata*) and black-winged (*Oncometopia nigricans* (Walker)) sharpshooters are native and ubiquitous, vine-to-vine, or within-field secondary spread of *X. fastidiosa* among grapes, occurs regularly throughout the growing season (1). Following the recent establishment of *H. coagulata* in portions of the southern San Joaquin Valley, the spatial patterns of PD incidence were described as nonrandom or occurring as large, elongate clusters of infected vines indicative of patterns of secondary spread that reflected the feeding patterns of the newly introduced vector (35).

In this study, the spatial patterns of ALS incidence, disease severity, and *X. fas-*

tidiosa genotype were nonrandomly distributed in only a portion of the almond orchards surveyed. The existence and degree of spatial dependence was influenced further by the particular almond cultivar evaluated. These differences may result from one or a combination of factors including dissimilar rates of overwinter survival, seasonal variations in bacterial populations, and feeding preference by xylophagous insect vector(s) (13,14,19). Our findings illustrate that disease incidence was greater in 'Sonora' and 'Carmel' than in the remaining cultivars, which were presumably less susceptible to or conversely more tolerant of infection by *X. fastidiosa*. Using geostatistics to illustrate the distribution of *X. fastidiosa* genotype, only G-genotypes of affected 'Sonora' trees at Kern County orchard 3 were observed to be clustered, while the remaining A-genotypes were randomly distributed (Fig. 6A and B). This is the first documented concomitant occurrence of multiple *X. fastidiosa* genotypes present in the same almond orchard. Moreover, with the diagnosis of PD in grapes adjoining orchard 3 (results not illustrated), these results further document the simultaneous association of both diseases in southern portions of the San Joaquin Valley where previously this occurrence was not reported. Almeida and Purcell (5) hypothesized that the rare cases of ALS in these areas were due to accidental infections by infective vectors emigrating from grape into susceptible almond. This pathway may help to partially explain the G-genotype aggregations observed in this study. However, further investigations are warranted to better understand the *X. fastidiosa* genotypes present within alfalfa, which occurs regularly in this region and flanked orchard 3 (and the adjoining PD-affected grape) on two borders.

The principal insect vectors of ALS have not been clearly identified in areas where this disease has been problematic historically, although presumably xylo-

phagous sharpshooters responsible for movement of PD into grapes (28,29) and numerous spittle bugs (33) are probable vectors. Our findings indicate that clusters of diseased trees were associated commonly with field borders adjoining habitats known to support populations of potential vectors. Primary spread of *X. fastidiosa* from outside inoculum sources would lead initially to random patterns of infected plants, which may or may not be followed by tree-to-tree movement, or secondary spread of the pathogen, resulting in disease clusters or foci. Over multiple seasons, successive waves of primary spread may account for the spatial patterns of ALS observed in our study where clusters of infected trees were often associated with field borders. Detailed surveys over successive seasons will be necessary to better understand the temporal patterns of ALS progress and the extent of secondary spread that may in fact occur. The occurrence and recent increase in incidence of ALS in the southern San Joaquin Valley of California does not directly reflect the introduction and establishment of the newly introduced vector, *H. coagulata*. This recently established vector species may, however, dramatically change the epidemiology of ALS in the future by exposing almonds to a more diverse population of *X. fastidiosa* and increasing the likelihood of secondary spread within orchards in mid- to late-summer when bacterial populations are highest (4).
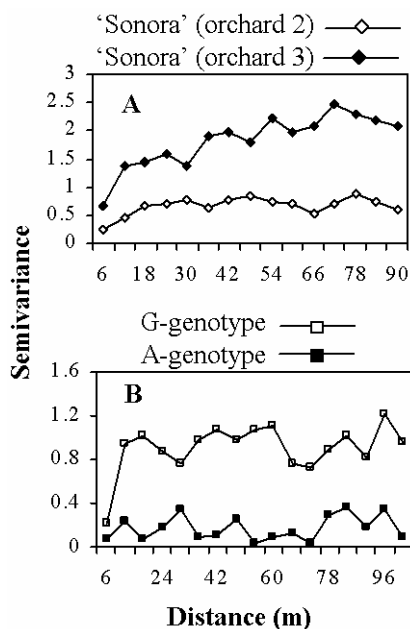
Fig. 5. Semivariogram plots of almond leaf scorch severity level = 4 within 'Sonora' in Fresno and Kern county survey orchards 2 and 3, respectively (**A**) and plots of *Xylella fastidiosa* genotypes within 'Sonora' in Kern County survey orchard 3 (**B**).
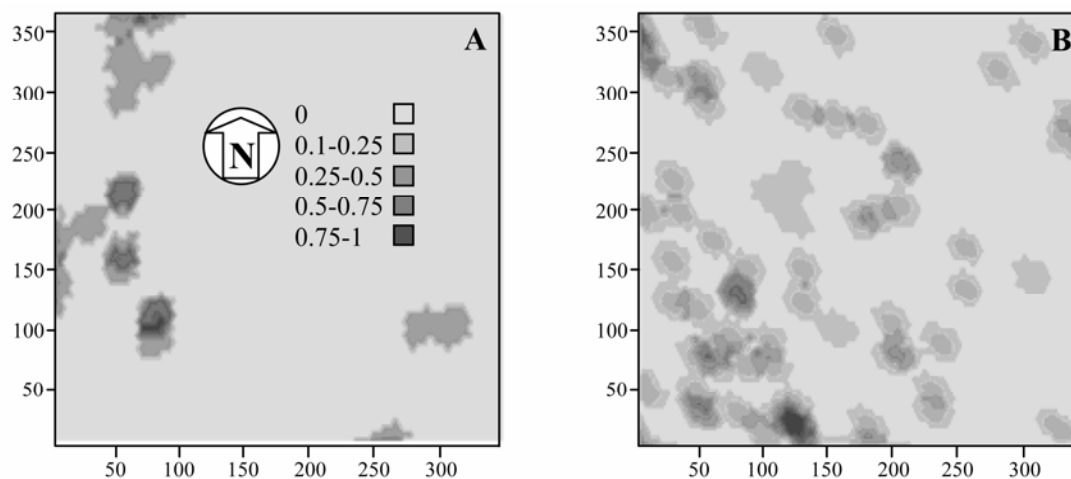
Fig. 6. Distribution of *Xylella fastidiosa* G-genotypes (**A**) and A-genotypes (**B**) using indicator kriging interpolations in Kern County survey orchard 3.

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